Engineering PON1 for Improved Stereoselectivity

Thesis

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Abstract

Organophosphorus nerve agents are harmful chemical substances that inhibit acetylcholinesterase activity. This impedes hydrolysis of the neurotransmitter acetylcholine at synapses in the brain and at neuromuscular junctions. Because of this, muscles in the body do not get the appropriate signal for muscle contraction. This leads to a number of side effects, including eventual death by asphyxiation. These chemical weapons have been used throughout modern history, including the use of Sarin in the 1995 subway terrorist attack in Tokyo by the Aum Shinrikyo religious group and more recently in Syria by rebel terrorist groups. These agents have been designed based on insecticides, specifically organophosphorus agents, because of their extreme toxicity toward higher vertebrae organisms. Civilians in many third world countries are fatally poisoned due to the toxicity of these insecticides. To provide protection against these insecticides and even organophosphorus nerve agents, Paraoxonase-1 (PON1) has been observed to have some catalytic efficiency for the agents. This esterase is found in serum at concentrations close to 50 μg/L, and has low activity to hydrolyze the more toxic isomer of these OP agents. Through directed evolution and protein engineering, a mutant of PON1 could be designed to have a high enough catalytic efficiency to hydrolyze the toxic isomer of these agents and eventually be administered to humans for protection.

Paraoxonase-1 is a 42 kDa calcium dependent esterase. It exhibits a six-bladed propeller tertiary structure with two calcium sites. In serum, PON1 is synthesized in the liver and circulates within HDL (high density lipoproteins) particles. Because it binds to HDL, it is suggested that PON1 may play an important role in atherosclerosis prevention and reverse cholesterol transport. PON1 primarily acts as a lactonase in vivo, but has

some activity for organophosphorus agents. Because of this, it is a novel candidate as a catalytic bioscavenger. Using directed evolution via gene shuffling of rabbit, human, mouse, and rat PON1 genes, the Weizmann Institute expressed chimeric recombinant PON1 enzyme (G3C9) in high yield in bacteria to use as a model for studying PON1 activity. This wildtype, G3C9, has a modest amount of activity towards these OPs and nerve agents. The organophosphorus agents such as GB, GD, VX, and VR all have stereoisomers and are synthesized as racemic mixture. Interestingly, the S_P-isomer of all these compounds is more toxic. G3C9 has little to no activity for the S_P-isomer of these molecules. However, through a number of rounds of directed evolution a mutant called I-1-F11 has been found to have a higher affinity for the S_P-isomer rather than the less toxic R_P-isomer, hydrolyzing the S_P-CMP-Coumarin analogue of CMP at a rate of 12.1 $\mu M^{-1}m^{-1}$. Because of this, I-1-F11 can be used as a model to improve the stereoselectivity of PON-1, which could eventually lead to an enzyme with close to diffusion limited activity towards the more toxic isomer of OP agents and insecticides. I-1-F11 differs by seven different residues from the wildtype, G3C9. These mutations include L55M, L69V, H115A, H134R, F222M, I291L, and T332S. By using site-directed mutagenesis of PON-1, the importance of these residues for stereoselectivity can be determined and other mutations significant for stereoselectivity can be identified. From our studies, all seven mutations appear to play a significant role in the stereoselectivity of 1-I-F11, especially L55M and L69V which have the most effect.

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Chapter 1

Introduction

1.1 OP compounds and nerve agent poisoning prevalence

Organophosphorus (OP) compounds have become some of the most prominently used pesticides over the past century due to their extreme level of toxicity. These compounds have been synthesized to increase stability and specificity for esterase inhibition in vivo, thereby obstructing many important biological functions and pathways.[1] Acute organophosphorus compound poisoning developed into a global health issue in the 1990's, with approximately 25 million episodes of intoxication occurring annually among agricultural workers alone.[2] Today, approximately 3 million of these incidents involve severe cases of poisoning, resulting in 220,000 deaths each year.[3] These incidents usually involve relatively high amounts of exposure when compared with nerve agents, which are OP compounds originally synthesized to inhibit acetylcholinesterase. The first synthesized OP compound, tabun, was developed by the German chemist Gerhard Schrader in 1936 as a chemical warfare agent with an LD_{50} of 4000 mg/individual.[4] Throughout WWII, other nerve agents such as soman and sarin were developed with even lower LD_{50}'s and thus posed more dangerous threats to the world's populations.[4] Reemergence of nerve agents, especially sarin, has been observed despite the U.N.'s banning of chemical warfare agent production at the Chemical Weapons Convention in 1993. On August 21, 2013, the U.N. investigated an incident near Damascus, Syria, in which hundreds of men, women, and children were killed.[5] For numerous research labs, identification of sarin gas and its degradation products presented a challenge previously tackled in Saddam Hussein's use of chemical weapons in Iraq

during the 1980s and the Tokyo subway attack involving sarin in 1995.[5] The current global prevalence of chemical weapon utilization as well as pesticide poisoning thus suggests a need for the development of a counteraction providing protection against these compounds. Our enzyme of interest, paraoxonase-1 or PON1, is one of the primary targets for providing protection from poisoning by mode of acetylcholinesterase inhibition.

1.2 Acetylcholinesterase biological function and action

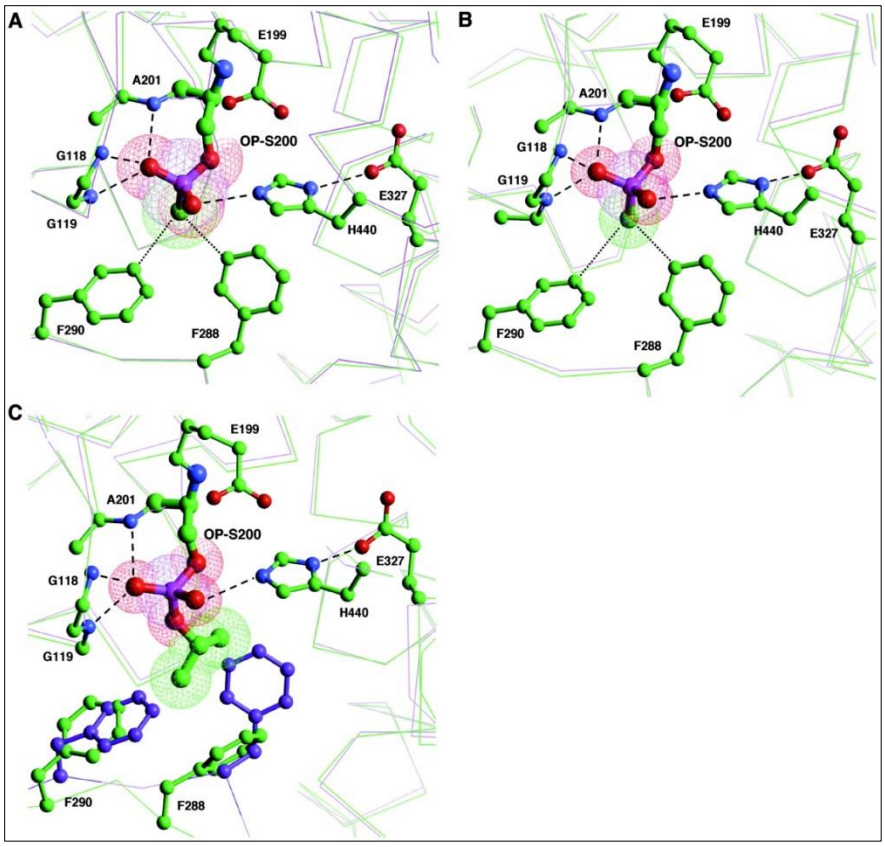
As stated previously, the primary mode of OP compound poisoning occurs via acetylcholinesterase inhibition. Acetylcholinesterase (AChE) is integral in biological movement and function, especially due to its role in the central and peripheral nervous systems. AChE is an extrinsic, membrane-bound enzyme that is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh).[6] In regularly functioning neurons, an action potential reaches the presynaptic nerve process causing ACh to diffuse across the synapse and bind to proximate ACh receptors. This leads to a cascade which opens K^+ and Na^+ channels in the adjacent axon or axons, eventually triggering an action potential in the postsynaptic neuron. To terminate this process, AChE hydrolyzes ACh into choline and acetate. Acetylcholinesterase possesses high specific activity that is diffusion limited, exhibiting a steady-state rate constant in the conversion of free enzyme and free substrate of 10^8 $M^{-1}s^{-1}$.[6] This aspect of acetylcholinesterase makes it an evolutionary "perfect" enzyme and is a product of its molecular structure.

1.3 Acetylcholinesterase structure-function relationship

The crystal structure of a homodimer of AChE (from the species *Torpedo californica*) form was solved using a hanging-drop vapor diffusion method and the presence of a catalytic triad consisting of residues S200, E327, and H440 has been confirmed (in mice, the residues have consist of S203, E334, and H447 respectively).[78] Also along these lines, recent Born—Oppenheimer ab initio QM/MM molecular dynamics simulations with umbrella samplings have been able to characterize AChE hydrolysis of ACh.[9] These studies show that the reaction is initiated by nucleophilic attack by the catalytic histidine, resulting in a high-energy tetrahedral covalent intermediate that is stabilized by an oxyanion hole formed by peptidic NH groups from G118, G119, and A201.[8] This reaction mechanism is impeded through inhibition by OP compounds. Nerve agents exhibit their toxicity by phosphonylating the catalytic serine of the serine side chain.[10] Typically, anticholinesterase OP compounds are either phosphoric or phosphonic acid derivatives and can be sulfur analogs. These compounds also usually consist of two alkyl substituents and a third substituent which acts as the leaving group.[11] After covalently bonding to the substrate, AChE is essentially irreversibly inhibited and shows slow to non-existent reactivation due to a process called aging. In this aging process, the dealkylation of the diakylphosphonyl enzyme results in a more stable monoalkylphosphonyl enzyme which has negligible rates of reactivation.[11] This mechanism has recently been supported through QM/MM studies with soman, showing an overall exothermic reaction in the formation of the stable "aged-AChE."[10]

Figure 1.1: Active site of aged phosphylated

TcAChE determined by X-ray crystallography[12]



1.4 AChE Inhibition Effects

Inhibition of AChE can have a number of physiological repercussions. Toxicity varies amount compounds, but typically only very low doses are needed to induce seizures and other related biological responses. When initially intoxicated, victims follow a three-phased process of poisoning. In general, these episodes are initiated by an early cholinergic phase which lasts from the onset of seizure and approximately five minutes afterward. This step is followed by a phase of mixed cholinergic/non-cholinergic modulation which continues for approximately 40 minutes, which is subsequently accompanied by a non-cholinergic phase.[13] In the case of human poisoning, symptoms include miosis, seating, rhinorrhea, lacrimation, salvation, abdominal cramps and other GI issues, respiratory difficulties, dyspnea, bradycardia, anorexia, nausea, vomiting,

diarrhea, involuntary urination and defecation, as well as damage to functions of the central nervous system which is associated with dizziness, confusion, and headaches. These symptoms are then typically followed by seizures, convulsions, mood swings, and other psychiatric issues. Death usually occurs due to respiratory failure in instances of OP poisoning.[14]

1.5 Stoichiometric sequestration of organophosphorus compounds

A number of methods have been developed to combat organophosphorus poisoning. Some of these methods are preventative, while others attempt to reactivate acetylcholinesterase after inactivation due to covalent binding of OP compounds to the catalytic site serine. Therapeutics designed for protection and/or biological function restoration are developed usually through one of three different methods: catalytic hydrolases, stoichiometric binding agents, or pseudo-catalytic treatments that use catalytic activity only in the presence of oximes.[15] Cholinesterases, and in particular butyrylcholinesterase (BChE), have been studied extensively as potential bioscavengers. HuBChE has proven to be capable of providing protection against a number of nerve agents such as tabun, sarin, soman, and VX via stoichiometric sequestration.[16–18] Recombinant human acetylcholinesterase has also been found to have similar to possibly higher levels of prophylactic protection, however only binding to the S_P-enantiomer of OPs.[19] Thus rHuAChE does not bind the R_P-enantiomer of these compounds, making BChE more efficient at sequestering a racemic mixture of OPs. In addition, rHuAChE is relatively unstable in human serum, but subsequent PEGylation displays a mean circulatory retention time of approximately 10,000 min.[1820] In addition to these

challenges, commercial and large scale production of AChE is still limited.[21] To stand a reasonable chance at providing protection *in vivo*, substantial stability becomes a necessity. Although the reported values vary, BChE demonstrates a half-life ranging somewhere between 44.7 h to approximately 10 days.[22] Whatever the case, BChE has sufficient stability to make it a viable drug when administered preventatively, although protection by these methods are solely through stoichiometric sequestration .[15]

1.6 Pseudo-catalytic and catalytic bioscavengers

Progress has also been made in the production and design of pseudo catalytic bioscavengers which have the capability to be regenerated, usually through oxime-mediated reactions. Because BChE has proven as the currently most viable option for stoichiometric sequestration of OPs, it is a primary area of research for pseudo catalytic treatments. Attempts at mutating BChE and AChE have been attempted to elicit catalytic hydrolysis from these previously stoichiometric bioscavengers, however most these efforts have failed or resulted in minimal actvivity.[21] Reactivation of paraoxon-inhibited AChE and BChE, as well as tabun-inhibited BChE, has been achieved through treatment with a number of different oximes.[23,24] A more attractive approach at this moment in time appears to be treatment with purely catalytic bioscavengers, possibly administered as a mixture containing supplementary stoichiometric bioscavengers if necessary. Some of these catalytic bioscavengers include but are not limited to phosphotriesterase (PTE), organophosphorus hydrolase (OPH), diisopropyl fluorophosphatase (DFP), Drp35, senescence marker protein 30 (SMP-30), and paraoxonase 1 (PON1).[25–28] Most of the research, however, has been conducted on SMP-30, DFPase, and PON1. From this point

onward, the focus of the text will be fixated upon PON1 pertaining to discovery, biological significance, and most importantly its potential as a bioscavenger.

1.7 PON1 discovery and physiological function

The discovery of PON1 is accredited to Dr. Abraham Mazur in 1946 when he was analyzing inhibition of plasma, red blood cells, and brain cholinesterases by diisopropyl flurophosphate within a number of tissue samples.[29] He came across the observation that rabbit plasma and human tissue samples have catalytic hydrolysis activity towards organophosphorus compounds, although very minimal activity. Today, PON1 today is known as a member of the paraoxonase gene family, consisting of members PON1, PON2, and PON3.[30] PON1 is a 43-45 kDa glycoprotein that is synthesized in the liver and then secreted into the serum where it circulates bound to high-density lipoprotein (HDL).[30,31] There is evidence that PON1 is implicated in atherosclerosis, possibly by protecting against low-density lipoprotein (LDL) oxidative stress by reducing macrophage foam cell formation and therefore reduction of atherosclerosis development.[32] A number of PON1 gene polymorphisms have been associated with a various human diseases, including coronary heart disease, Parkinson's disease, type 2 diabetes, and inflammatory bowel disease.[33–35] It is believed that the primary biological function of PON1 is its capacity to hydrolyze lipid peroxides, preventing foam cell formation and hence atherosclerosis,[32] however work is still being done on the subject. Because it is already present within the human body in serum, PON1 presents a viable option as a bioscavenger with a potentially negligible immunogenic response, thus making it advantageous in drug delivery. In the attempts of designing PON1 as a

potential bioscavenger, one must consider humanization or *in vivo* stability, catalytic efficiency, and substrate specificity.

1.8 PON1 structure

There is a significant amount of literature on mutagenesis of PON1 aiming to increase catalytic efficiency, however the exact mechanism of its reactivity is still unclear. In 2004, the first crystal structure of PON1 was solved giving some insight into a possible mode of reactivity. Through directed evolution using gene shuffling from human, rabbit, mouse, and rat PON1 sequences, the variant G2E6 was crystalized.[31]

Figure 1.2: Serum Paraoxonase by Directed Evolution[31]

This mutant, having 91% homology with WT rabbit PON1, has mutations that presumably do not interfere with the active site of the protein. From the crystal structure,

it was determined that PON1 is a six-bladded β-propeller containing one disulfide bridge and two calcium ions within the core.[31] The tertiary structure PON1 resembles that of a number of other enzymes, including diisopropylfluorophosphatase and SMP-30.[36,37] It is also believed that the hydrophobic N-terminal signal peptide is necessary for association with HDL *in vivo* and is also responsible for binding to the lipoprotein molecule.[38] The binding mode of PON1 to HDL is not entirely understood, however Apolipoprotein A-I stabilizes PON1 *in vitro* and could quite possibly be implicated in PON1 and HDL interactions. [38] Although the structure of PON1 has been solved, the mechanism of PON1's enzymatic activity is still unknown and will be the focal point of this discussion.

1.9 PON1 substrate promiscuity

Investigation of the mechanism of PON1 has led some to believe that PON1 was selected by nature as a lactonase in a recent paper looking at the evolution of the three mammalian PON families.[39] Its possible physiological substrate could be homocysteine thiolactone as it is in the only known molecule with homothcystrine thiolactonase activity found within the body. PON1 also exhibits excellent hydrolysis activity towards phenyl acetate with a k_{cat}/K_M of 10^6 M^{-1} s^{-1}.[40] Most importantly, PON1 has some phosphotriesterase activity, although mostly likely did not evolve as a phosphotriesterase because these substrates are synthetic and have only been in existence on Earth for the past several decades.[40] HuPON1exhibits differences in activity toward a number of substrates (such as V-type nerve agents, phenyl acetate, and paraoxon) in comparison with G2E6 and rabbit PON1 (G3C9).[41] These discrepancies suggest that even though the variation in residues did not occur within the active site itself, effects on activity were

still observable and that the global orientation of the protein was altered.[41] Figure 1.9 demonstrates the 3 primary modes of activity of PON1 with reactions a, b, and c which correlate to lactonase, esterase, and phosphotriesterase activity respectively.[42]

Figure 1.3: Catalytic versatility of Paraoxonase 1[42]

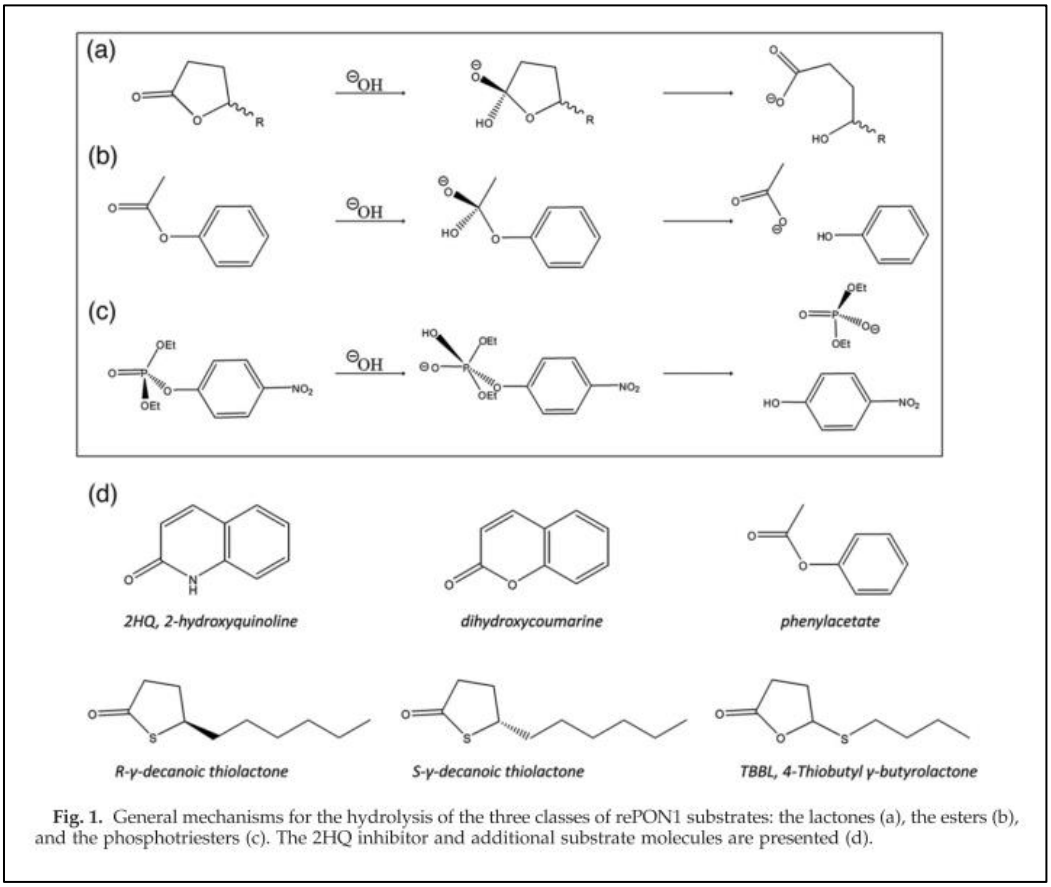


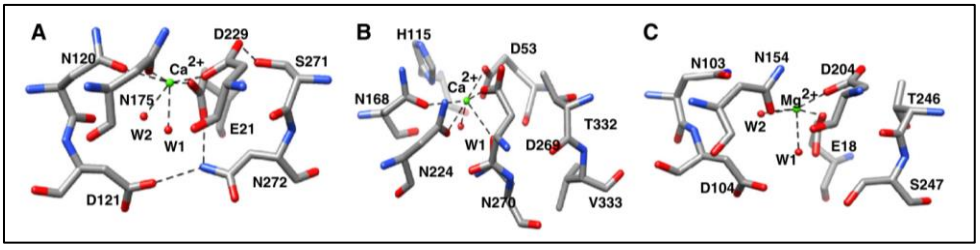
Fig. 1. General mechanisms for the hydrolysis of the three classes of rePON1 substrates: the lactones (a), the esters (b), and the phosphotriesters (c). The 2HQ inhibitor and additional substrate molecules are presented (d).

1.10 PON1 catalytic mechanism

The promiscuity of PON1's enzymatic activity alludes to the mystery of its catalytic mechanism. It is generally accepted that the active site of the protein is located at the upper calcium.[31] The exact active site reside, however, has not been identified. Originally, it was believed that His134 within the active site acts as a proton acceptor to increase the basicity of His115, forming a His-His dyad in which His115 activates a

water molecule through deprotonation which then serves as the nucleophile within the reaction.[31] However, recent variants of PON1 simultaneously lacking histidine at both 115 and 134 still exhibit lactonase/arylesterase activity and thus it has been proposed that there are multiple residues within the active site that are capable of producing hydrolytic activity.[43] Some have tried to investigate enzymes with similar structure and function, including SMP-30 and DFPase, to demystify the mode of activity. By superimposing the three enzymes upon each other, similarities in the binding pocket and metal ion geometry, flexible loop position and implication, and β-sheet blade locations are all observable.[37] DFPase, PON1, and SMP-30share a flexible loop near the active site. In PON1, it is theorized that movement of this loop is involved with substrate binding by moving in and out of the active site.[37] As for the geometry of the active site, the residues responsible for metal ion binding are relatively well conserved. In PON1, the ligands include E53, N168, N224, N270, and D269. Figure 1.4 illustrates the metal-binding residues of all three enzymes, showing the similarities between all three.

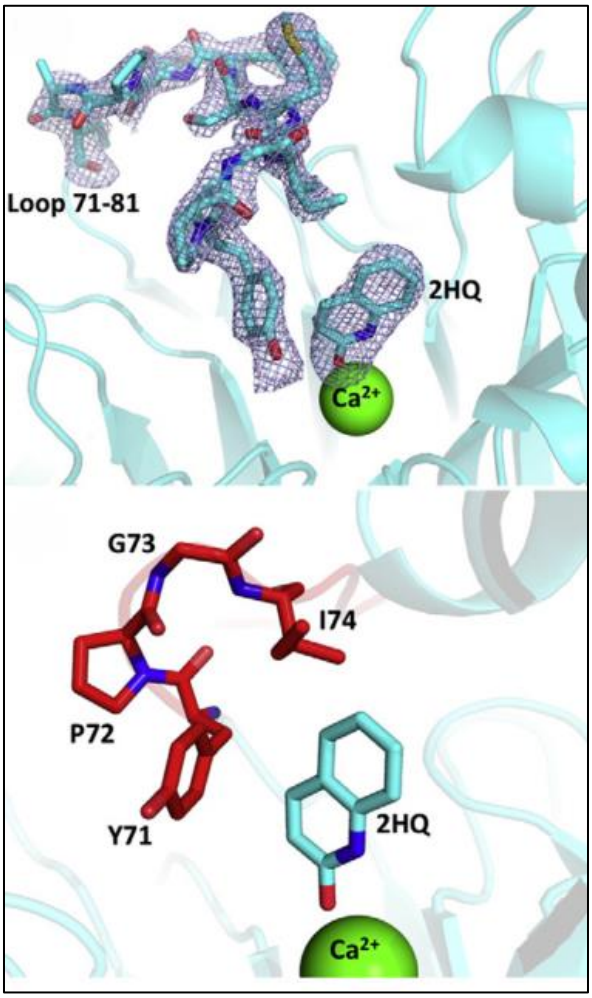
Figure 1.4: Comparison of active sites of DFPase, Hu PON1, and Hu SMP-30[37]



The most significant difference between PON1 and the other two enzymes are the subpockets of the active site, consisting of subpockets 1 and 2. The first subpocket in PON1 is composed of residues L69, F347, and H115, while the second subpocket includes residues F222, L240, L267, and F292. The secondary hydrophobic pocket

allows for substrate specificity due to its high hydrophobicity, thus providing stability for a myriad of substrates with large hydrophobic groups in comparison to DFPase and SMP-30. More recent studies of PON1 suggest an alternative hypothesis for the mechanism of PON1 activity. A more recent crystal structure of rePON1 has been solved at a new pH of 6.5, rather than the previous 4.5 pH of the past structure, giving further insight into the enzymatic activity of PON1.[42] In the crystal structure, PON1 inhibitor 2HQ (2-hydroxyquinoline) is bound to the active site calcium.

Figure 1.5: 2HQ within the active site[42]



In figure 1.5, one can see that the flexible loop containing residues 71-81 lies above the

active site. This loop can be anchored to the surface of the enzyme where Y71 points into the active site. In this state, PON1 is considered to be in the closed conformation. In the open conformation, the active site flexible loop is unanchored from the surface of protein and moves freely.[42] Because this crystal structure was obtained in the closed conformation with 2HQ bound in the active site, it is believed that during substrate binding the flexible loop "closes" overtop of the active site of PON1. Docking studies using a variety of substrates were then performed assuming this closed conformation.

Figure 1.6: Molecular docking of PON1 variant G2E6 with various substrates[42]

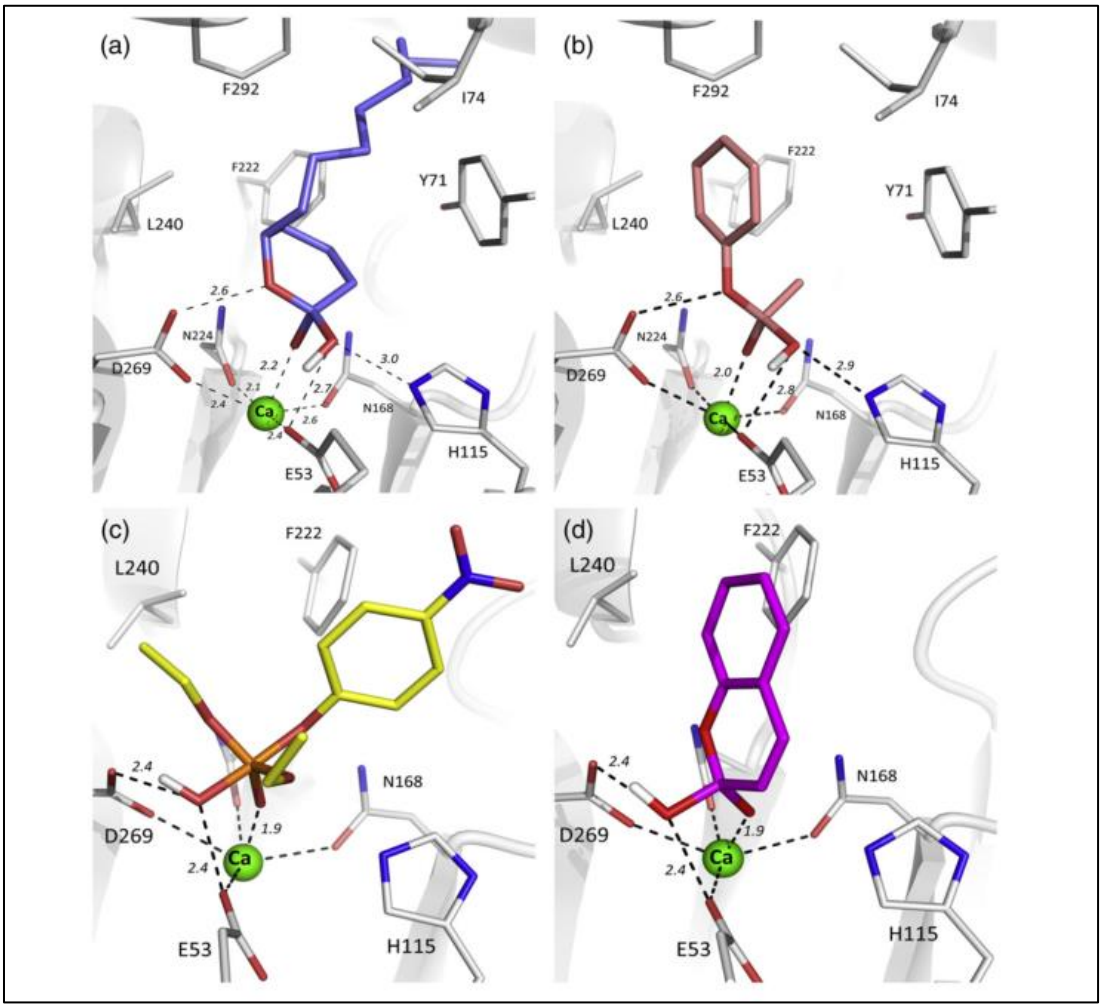
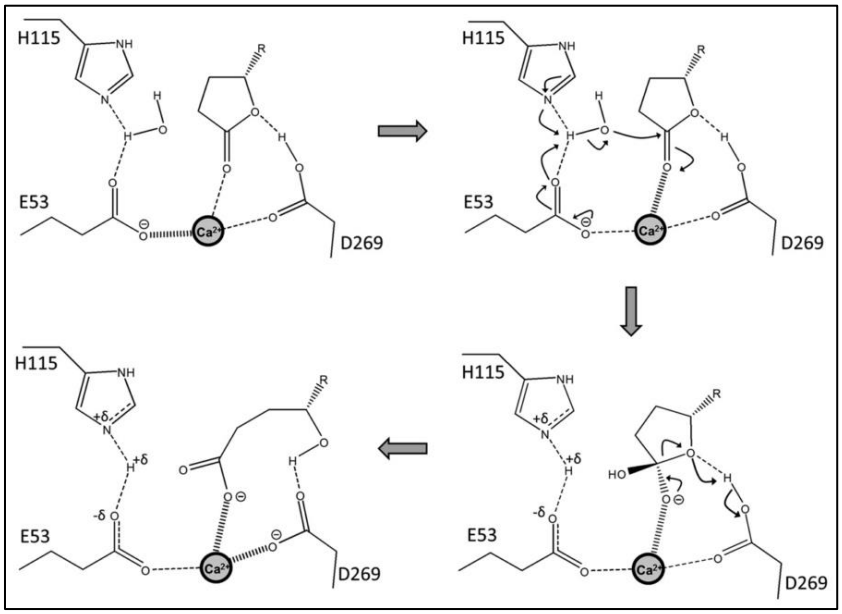


Figure 1.5 illustrates substrate binding with the S-isomer of γ-nonanoic lactone (a),

phenylacetate (b), paraoxon (c), and dihydrocoumarin (d). While docking γ-nonanoic lactone, phentylacetate, and dihydrocoumarin with PON1 in closed conformation were successful, attempts at docking paraoxon within the active site in the closed conformation were not. Y71 is the principal residue involved in inhibiting the binding of PON1 in this confirmation, however when mutated to a smaller residue such as alanine, paraoxon hydrolysis activity is significantly decreased. It is thus believed that binding of paraoxon within the active site occurs in the open confirmation.[42] Also, when docking these substrates within the active site, there appears to be differences between the primary residues interacting with the substrates. Phenylacetate and γ-nonanoic lactone display close interactions with H115, while paraoxon and dihydrocoumarin show interactions with residues E53 and D268. Currently, the most accepted mechanism of PON1 activity depends upon the substrate. The theorized lactonase activity is depicted in figure 1.7.

Figure 1.7: Hydrolysis of lactones by rePON1



Under this model, a hydroxide nucleophile is generated via general base catalysis by E53

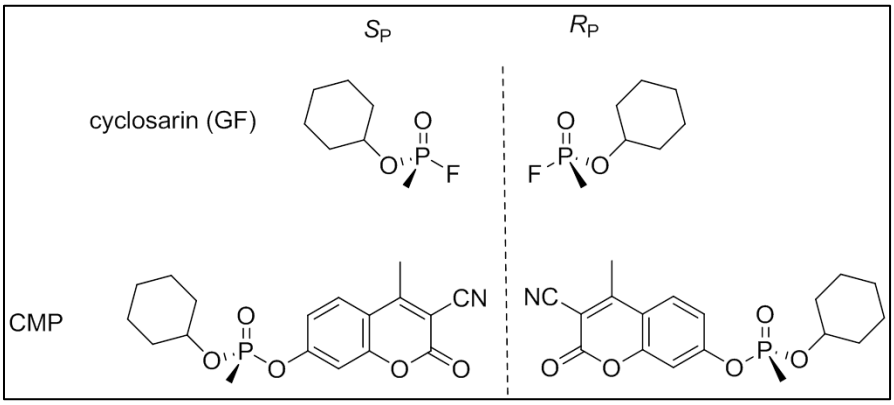
and H115 in regards to lactonase activity. With respect to paraoxon and dihydrocoumarin, an alternative mechanism must be considered due to absence of effects on activity when mutating out H115. Proposed mechanisms include nucleophilic attack by the side chains of E53, D269, or other residues within the active site. Another possible mode of catalysis could be water activation by E53 or D269.[42] Along with the issues in understanding the mechanism of PON1, another challenge arises when analyzing the mechanistic properties. Namely, the stereochemistry involved in these reactions becomes relevant if PON1 is to be used as a bioscavenger.

1.11 PON1 stereoselectivity

As stated previously, AChE binds preferentially to the S_P isomer of OPs.[19] Past attempts at improving the catalytic efficiency of PON1 has been unsuccessful in selecting for activity towards the S_p isomer of these compounds until recently.[44–46] Directed evolution using high-throughput fluorescence-activated cell sorting (FACS) and low-throughput plate screening has been utilized to improve catalytic efficiency relatively rapidly while screening against various nerve agents.[46] By screening against the stereoisomers of these compounds, mutations can be selected to improve preferential reactivity for the S_P isomer. Through this process, a number of variants have been designed with improved reactivity for the S_P isomer of G-type agents.[45,46] G-type nerve agents such as tabun (GA), sarin (GB), soman (GD) and cyclosarin (GF) are some of the most highly toxic organophosphates, penetrating the body by either inhalation or skin absorption. These OPs have LD_{50}'s ranging from approximately 0.071-0.714 mg/kg;[45,47] according to theorized models, prophylactic activity on the order of ~10^7 M^{-1} min^{-1} would

be necessary for protection against OPs of such extreme toxicity. In particular, the PON1 variant 1-I-F11 was found after 3 rounds of screening starting from the variant 2D8. 2D8 contains the mutations L69G, S111T, H115W, H134R, F222S, and T332S from WT PON1, G3C9.[46] Mutant 1-I-F11 contains the mutations L55M, L69V, H115A, H134R, F222M, I291L, and T332S and has an activity of 12.1 • 10^6 M^{-1} min^{-1} towards the S_p isomer of CMP-coumarin, the coumarin analog of GF.[45] Figure 1.8 depicts S_P and R_P isomers of both GF and CMP.

Figure 1.8: G-type nerve agent stereochemistry



WT PON1 has negligible catalytic activity for this substrate, either the S_p or R_P isomer, whereas 1-I-F11 has an extremely high activity for the S_P isomer and essentially no activity for the R_P isomer with a 2575 fold difference in k_{cat}/K_M.[45] The exact reason behind this inversion in reactivity for the S_P isomer is not entirely understood, although it has been theorized that the combination of mutations I291L and T332S play a crucial role in this specificity. From this point, we attempted to understand the stereoselectivity of the variant 1-I-F11 through various methods.

Chapter 2

Objectives

2.1 Design and study of PON1 chimeric mutants

1-I-F11 differs from WT PON1 (G3C9) by seven residues near the active site of the enzyme. No crystal structure of 1-I-F11 currently exists, so mutagenesis and analysis of variants with enzyme kinetics will be implemented to understand the stereoselectivity of the variant for the S_p isomer of OPs. This presents a challenge of selecting what mutations to make, such as single mutations, pairs, or even groups of mutations. One could potentially design thousands of variants with any one of these sets of mutations, so a rational approach must be taken in deciding what route of mutagenesis to implement. The first step of this project was to analyze a group of mutations from 1-I-F11 to determine if only a certain few residues are responsible for stereoselectivity before making individual mutations to 1-I-F11.

2.2 Design and study of 1-I-F11 single point mutants

It is possible that a single residue can have significant implications on the stereoselectivity of the enzyme. Singe point mutants of 1-I-F11 could be engineered by reverting the seven mutated residues back to the G3C9 amino acid at that position. Single mutations of PON1 would allow for easier analysis of changes in catalytic activity and possibly more insight into the mechanism of PON1 activity, especially the stereoselectivity for the S_p isomer of CMP found in the PON1 variant 1-I-F11.

Chapter 3

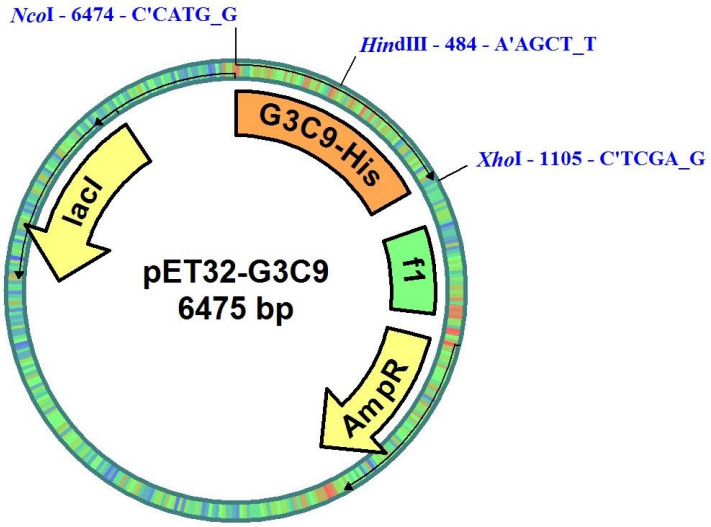
Materials and Methods

3.1 Design and study of PON1 chimeric mutants

Gene construction of chimeric mutants

G3C9 and 1-I-F11 constructs were obtained from our collaborator Dan S. Tawfik at the Wiezmann Institute. To investigate a group of mutations, our rationale was to divide the mutations according to DNA sequence for ease and uncertainty in what group of mutations to select. A location between residues 134 and 222 thus seemed appropriate, as one could design two chimeric mutants by splitting the mutations up at this location. Below is a plasmid map of the G3C9 plasmid along with selected restriction sites.

Figure 3.1: G3C9 plasmid map



In the digestion reaction was 50 µL of G3C9 or 1-I-F11 miniprep, 3 µL of both enzymes, 10 µL of NEBuffer #4 (New England BioLabs, Inc.), and 5 µL of 18MΩ-cm water. The

reaction was allowed to persist for 2 hours and was subsequently loaded onto a 1% agarose DNA gel. The insert and vector bands were cut from the gel and then gel purified following QIAquick Gel Extraction Kit Protocol. After gel purification, the insert from the G3C9 plasmid was ligated into the remaining 1-I-F11 vector; likewise, the 1-I-F11 insert was ligated into the remaining G3C9 vector. These ligations were performed using 1:5 and 1:10 ratios of insert to vector based on size of the DNA fragments and 1 µL of T4 DNA ligase with T4 DNA ligase buffer (New England BioLabs, Inc.).The ligation reactions were carried out at 16 ºC overnight and then transformed into DH10B *E. coli* cells via electroporation. Cells were plated onto LB agarose plates with ampicillin, grown overnight at 37 ºC, and then stored at 4 ºC. Individual colonies were picked and minipreps following QIAprep Spin MiniPrep Protocol were performed. Samples were then sent to GENEWIZ, Inc. for sequencing to confirm that the correct sequence had been produced. These two new variants were named 1-I-F11-G3C9 and G3C9-1-I-F11, in which each contained the following amino residues at the relevant positions with the WT residues listed in red.

Table 3.1: Chimeric variants with corresponding mutations

Variant	Residues
1-I-F11-G3C9	M55, V69, A115, R134, F222, I291, T332
G3C9-1-I-F11	L55, L69, H115, H134, M222, L291, S332

3.2 Design and study of 1-I-F11 single point mutants

Gene construction of single point mutants

Selected amino acids were replaced by site-directed mutagenesis of the 1-I-F11 gene using QuikChange Site-Directed Mutagenesis protocol. Below is a list of the

primers designed for all of the single point mutations of 1-I-F11:

Table 3.2: Site-directed mutagenesis primers for single point mutants

Variant	Forward Primer	Reverse Primer
1-I-F11-M55L	GAC AAT GGT TCT GAA GAC TTA GAA ATA CTG CCC AAT GGA CTG	CAG TCC ATT GGG CAG TAT TTC TAA GTC TTC AGA ACC ATT GTC
1-I-F11-V69L	GCT TTC ATC AGC TCC GGA CTC AAG TAT CCT GGA ATA ATG	CAT TAT TCC AGG ATA CTT GAG TCC GGA GCT GAT GAA AGC
1-I-F11-A115H	GAT ATA TCT TCA TTT AAC CCT CAC GGG ATT AGC ACA TTC	GAA TGT GCT AAT CCC GTG AGG GTT AAA TGA AGA TAT ATC
1-I-F11-R134H	CTA CTG GTG GTA AAC CAC CCA GAC TCC TCG TCC ACC	GGT GGA CGA GGA GTC TGG GTG GTT TAC CAC CAG TAG
1-I-F11-M222F	GCA GAA GGA TTT GAT TTC GCT AAC GGA ATC AAC	GTT GAT TCC GTT AGC GAA ATC AAA TCC TTC TGC
1-I-F11-L291I	CCC AAC GGA ATG CGA ATC TTC TAC TAT GAC CCA AAG	CTT TGG GTC ATA GTA GAA GAT TCG CAT TCC GTT GGG
1-I-F11-S332T	GTA TTA CAG GGC AGC ACA GTG GCC GCT GTG TAC	GTA CAC AGC GGC CAC TGT GCT GCC CTG TAA TAC

Each PCR reaction was digested with DpnI overnight before transformation into DH10B cells. All the samples were sent for sequencing to confirm successful mutagenesis.

1-I-F11-HA tag cloning

To improve the sensitivity of samples for *in vivo testing*, a hemagglutinin (HA) tag was introduced on the C terminus of the 1-I-F11 gene with the following primers:

Forward primer: GGTGGTGGTCCATG GCTAAACTGACAGCGCTCAC

Reverse primer: GGG GCC GCA CTT GAG CAC CAC CAC CAC CAC CAC TAC CCA TAC GAC GTC CCA GAC TAC GCT TGA GAT CCG CTC GAG AAA CCC.

After successful ligation, the samples were sent for sequencing.

Blank mutant gene construction

After calculating activities of all variants toward racemic CMP and S_P-CMP, we decided to construct another mutant of 1-I-F11 containing the mutations M55L, V69L, and A115H. Double digestions of both G3C9 and 1-I-F11 were performed with the restriction enzymes NcoI and BseRI. The insert from the G3C9 digestion was then ligated into the remaining 1-I-F11 vector and the samples were sent for sequencing to ensure proper ligation.

Protein expression and purification

All PON1 variants were overexpressed in BL21 (DE3) from colonies or glycerol stocks in 1 L 2YT media containing 1 mM $CaCl_2$. Cells were grown at 37 ºC until log phase, which occurred at approximately 0.8 OD at 600 nm, and were then induced to 0.1 mM IPTG while lowering the temperature to 30 ºC for 4 h. Cell pellets were harvested after spinning the cell cultures at 5000 rpm for 10 min at 4 ºC and stored at -80 ºC. For purification, all steps were carried out at 4 ºC . were then resuspended in lysis buffer (50 mM Tris pH 8, 50 mM NaCl, 1 mM $CaCl_2$, 1 mM BME) in approximately 10 volumes of total solution to 1 volume of cell pellet. After cells were lysed via sonification, 0.1% tergitol was added to the cell lysis and incubated for 2.5 h while shaking at 4 ºC. Cell

lysis was then centrifuged at 12,000 RPM for 30 min at 4 ºC and the supernatant was transferred to a clean 50 mL falcon tube. Approximately 2-3 mL of Ni-NTA slurry (Qiagen) was washed with activity buffer (50 mM Tris pH 8, 50 mM NaCl, 1 mM $CaCl_2$, 1 mM BME, 0.1% tergitol) plus 10 mM imidazole and then added to the supernatant to incubate while shaking on a Nutator for 3 h at 4 ºC. The resin was then loaded on a washed 25 mL column and the unbound material was allowed to flow through the column. The resin was then washed with 50 mL of wash buffer 1 (activity buffer containing 10 mM imidazole) and 15 mL of wash buffer 2 (activity buffer containing 25 mM imidazole). The desired PON1 bound to the Ni-NTA resin due to the 6×His-tag was then eluted with 30 mL of elution buffer (activity buffer containing 150 mM imidazole). Samples collected from protein purification were analyzed with SDS-PAGE (12.5% agarose). Fractions containing PON1 were dialyzed into activity buffer with 10% glycerol 2 h followed by dialysis into activity buffer with 50% glycerol for 3 h. Concentrations of PON1 were then determined with Bradford protein assay, which was confirmed visually using SDS-PAGE.

Western blot

Purified protein with variable concentrations mixed with SDS loading buffer were heated at 95 ºC for 10 min. Samples were loaded and run on a 12.5% SDS-PAGE gel and transferred to a nitrocellulose membrane (GE healthcare). After blocking with BSA for 1 h at room temperature, the blots were incubated overnight at 4 ºC with anti HA-tag antibody (1:5000 dil) (Millipore). The membrane was subsequently incubated with a secondary antibody, goat anti mouse IgG (1:200 dil) conjugated to horseradish

peroxidase and developed using a chemiluminescent detection system followed by scanning using a Typhoon (GE lifescience). 1-I-F11 WT without HA tag was used as a control.

Enzyme kinetics with racemic CMP

CMP was synthesized by the Hadad group as a racemate and dissolved into methanol. To assess concentration of dissolved substrate, CMP was hydrolyzed with the PON1 variant 3B3 which turns over only the R_P isomer of CMP, thus hydrolyzing half of the total amount of CMP in solution[46]. The purified variants were then tested for their activity against racemic CMP. First, 186 µL of assay buffer (50 mM Tris pH 8, 10 mM $CaCl_2$) and 4 µL racemic CMP were added to a well of a 96-well microassay plate, after which 10 µL of appropriated diluted enzyme was added to the well and immediately placed into an M5 spectrophotometer and then plotted absorbance versus time. The slope was measured within the linear portion of each trial where it was assumed the substrate-enzyme complex remained constant. All kinetic parameters were collected by Michaelis-Menten steady-state kinetics. A Michaelis-Menten plot can be made by plotting the slope obtained from the change in absorbance over time (mM min^{-1}) versus concentration of the substrate in each reaction.

Figure 3.2: Michaelis-Menten equation

$$v_0 = \frac{V_{max}[S]}{K_M + [S]}$$

Above is the Michaelis-Menten equation where V_{max} is $k_{cat}[E]_t$. Experimentally, K_M is the concentration of substrate at half V_{max}. V_{max} is the horizontal asymptote of the curve. If

the concentration of substrate is much smaller than K_M, it is possible to extract the enzymatic efficiency (k_{cat}/K_M) by simply dividing the rate by the substrate and enzyme concentration. However, if the substrate concentration is much larger than the K_M value, it is possible to obtain the catalytic activity (k_{cat}) of the enzyme by dividing the rate by the enzyme concentration. The k_{cat}/K_M is the most common value use in reporting enzymatic activity. Each variant was tested against four different concentrations of racemic CMP and the k_{cat}/K_M was determined by plotting absorbance per min versus concentration of racemic CMP by using the known extinction coefficient (ε) equal to 37450 M^{-1} cm^{-1} for CMP.

Enzyme kinetics with S_P-CMP

To prepare S_P-CMP, racemic CMP was hydrolyzed with the PON1 variant 3B3 overnight to ensure complete hydrolysis of R_P-CMP. This reaction contained 181 µL assay buffer, 4 µL CMP, and 5 µL diluted 3B3. After overnight incubation, PON1 variants were reacted with the remaining S_P-CMP by adding 10 µL of PON1 to 190 µL of the reaction mixture. Activity towards S_P-CMP was calculated using single point kinetics.

Molecular docking with S_P-CMP

Molecular docking was performed by Kate Cahill from the Hadad research group using AutoDock. Images of S_P-CMP bound to the calcium within the active site were obtained to help visualize the effects of the 1-I-F11 mutations.

Chapter 4

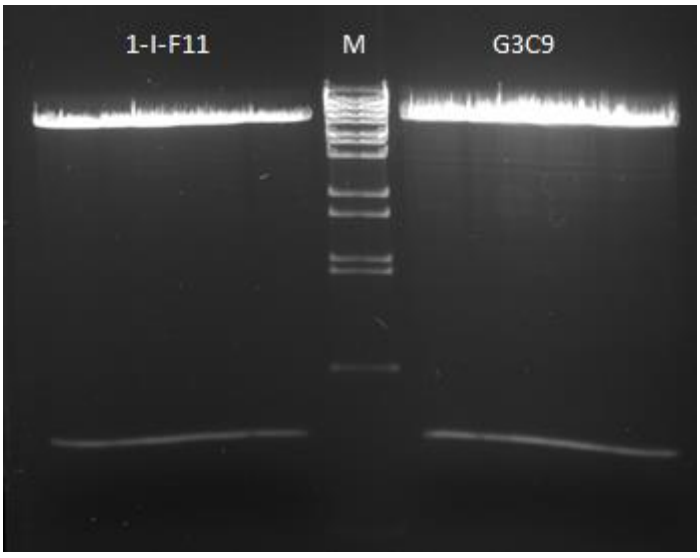
Discussion and Results

4.1 Design and study of PON1 chimeric mutants

I-F11-G3C9 and G3C9-I-F11 gene construction

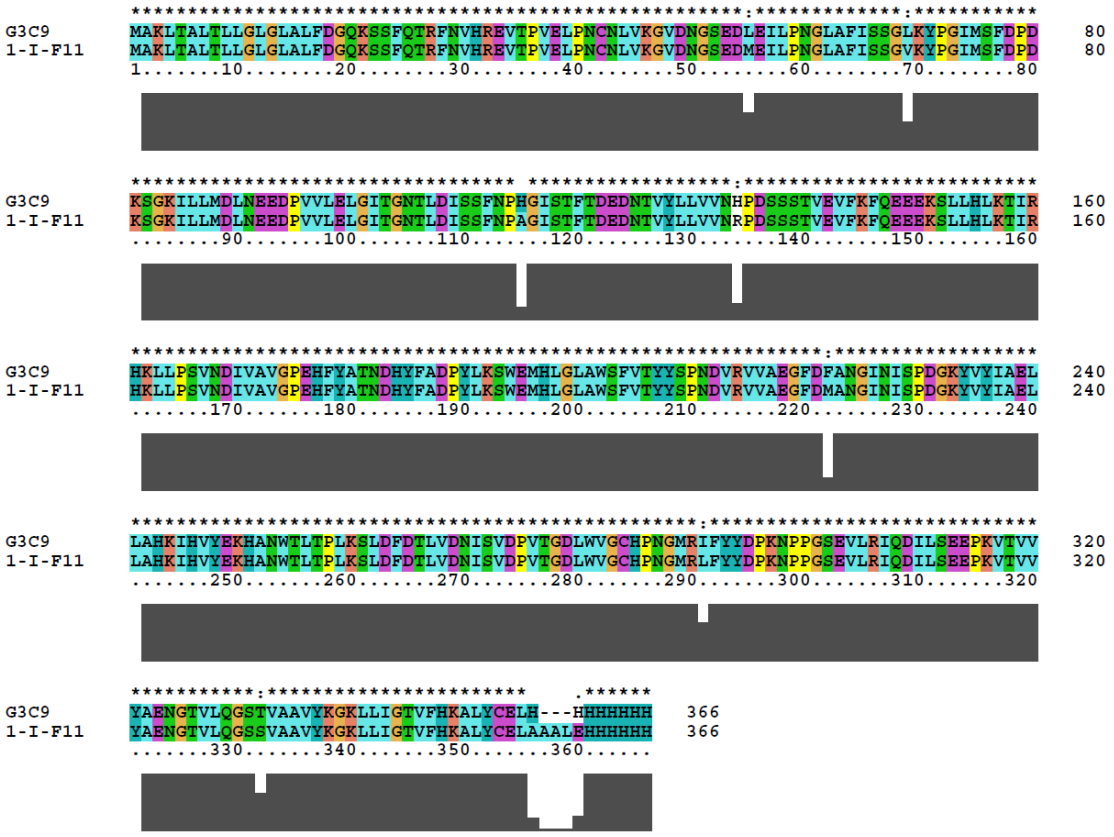
To swap the N-terminal portions of 1-I-F11 and G3C9, the digested inserts (Fig. 4.1) were ligated into the appropriate vector and the samples were confirmed by sequencing.

Figure 4.1: Double restriction digest of 1-I-F11 and G3C9



The sequences were aligned (Fig. 4.2) to confirm the correct sequence of all new variants.

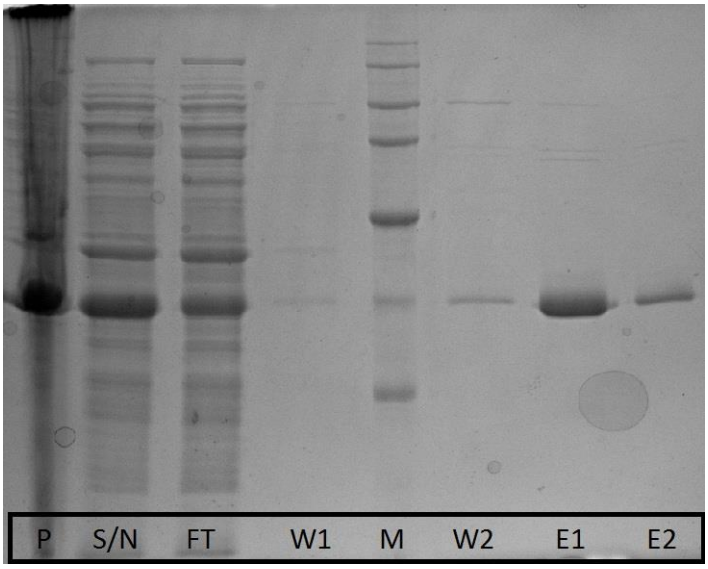
Figure 4.2: Sequence alignment using ClustalW2



Expression and purification

All variants were expressed similarly to the WT. No changes were observed in the yield of product. The following gel is a good indication of the yield achieved from the purification of each variant.

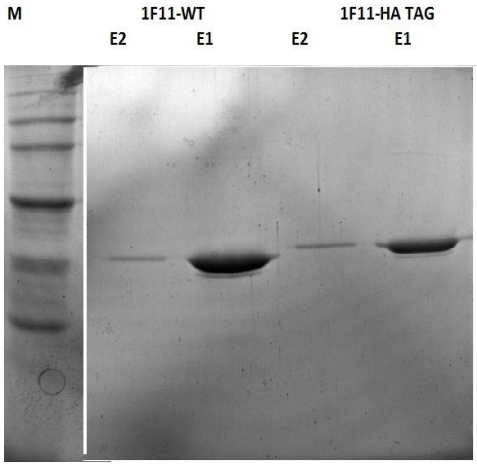
Figure 4.3: G3C9 purification



1-I-F11-HA tag and western blot

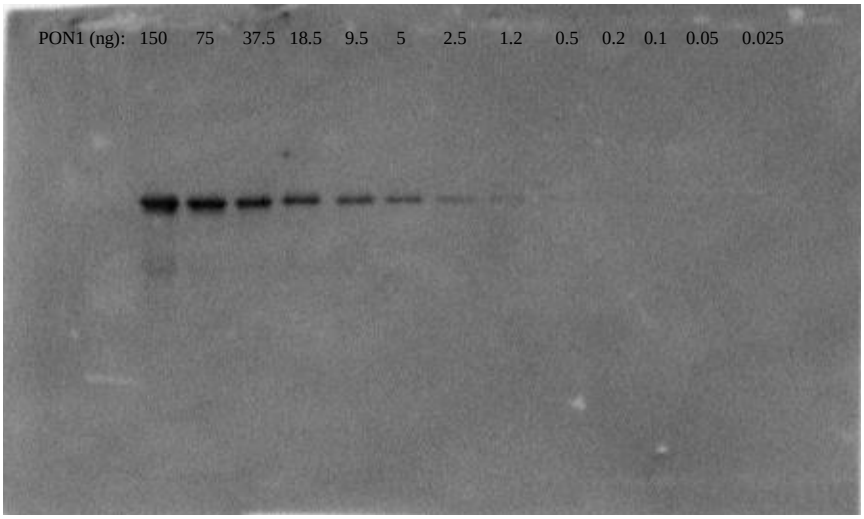
To overcome detection limitations of the CMP assay with *in vivo* studies, an HA tag was introduced on the C terminus of 1-I-F11 and the sequence was confirmed. The 1-I-F11 HA tag variant expressed well (Fig 4.4).

Figure 4.4: 1-I-F11-HA tag purification



After successful purification, we wanted to determine the lowest possible limit of protein detection by Western blot. From the following gel, the lowest possible protein amount we can detect from Western blot using HA tag antibody is approximately 2 ng (Fig 4.5).

Figure 4.5: 1-I-F11-HA tag Western blot



1-I-F11-HA tag kinetics

We wanted to see whether the HA tag had any effect on the activity of 1-I-F11. After performing kinetics on 1-I-F11-HA tag, a k_{cat}/K_M of 7000 mM^{-1} min^{-1} against racemic CMP was calculated. Compared to the k_{cat}/K_M of 15000 mM^{-1} min^{-1} against racemic CMP of 1-I-F11 WT, about a 2 fold decrease was observed. This was small enough of a change in activity to allow for *in vivo* studies to be conducted with 1-I-F11-HA tag.

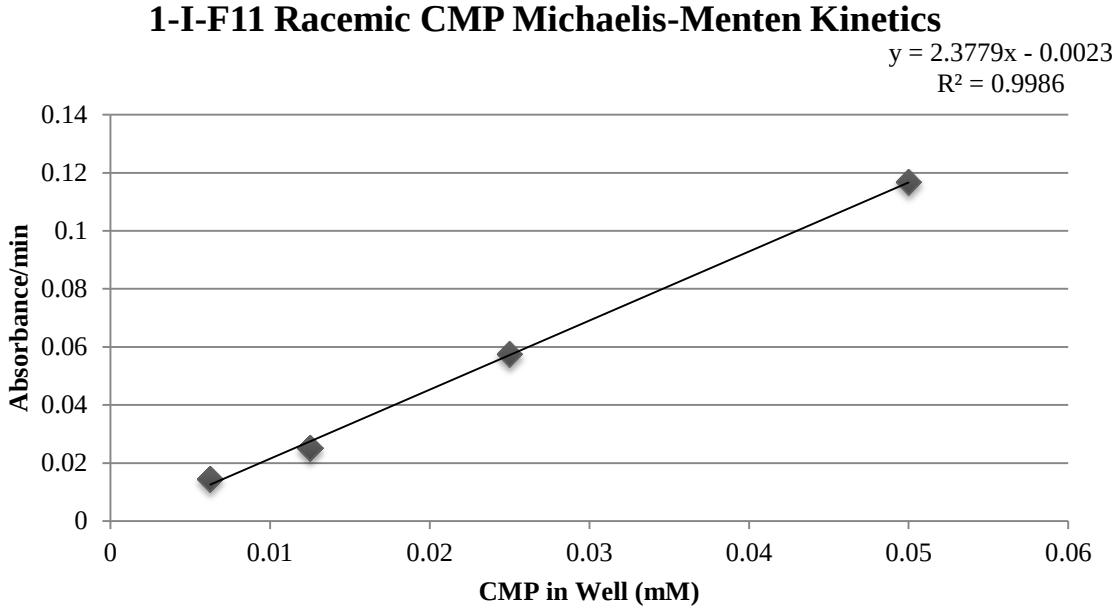
Chimeric mutant kinetics

Table 4.1: CMP activity summary of chimeric variants

Mutant	**S_P-CMP** ($mM^{-1}min^{-1}$)	**Racemic CMP** ($mM^{-1}min^{-1}$)	**R_P-CMP** ($mM^{-1}min^{-1}$)	**R_P/S_P**
1-I-F11	4200	15000	10800	2.6
I-F11-G3C9	50	2300	2250	50
G3C9	3.8	1020	1016	270
G3C9-I-F11	3.6	150	146.4	40.7

Table 4.1 illustrates the activities of G3C9, 1-I-F11, and the two chimeric mutations. Below (Fig. 4.4) is a depiction of a typical Michaelis-Menten plot.

Figure 4.6: Michaelis-Menten plot



R_P-CMP was calculated by simply subtracting the activity against S_P-CMP from the activity against racemic CMP. From the results, one can see a drastic loss in stereoselectivity for S_P-CMP in both chimeras. The N-terminal mutations of M55L, V69L, A115H, and R134H in G3C9-I-F11 appear to have both a significant impact on the activity against both racemic CMP and S_P-CMP. However, G3C9-IF11 still maintains a similar ratio of R_P-CMP to S_P-CMP activity in comparison to I-F11-G3C9. Thus, both sets of mutations are important for the stereoselectivity of PON1. Specifically, the residues in M55, V69, A115, and R134 appear to be more influential in the activity of PON1 towards CMP when compared to M222, L291, and S332.

4.2 Design and study of 1-I-F11 single point mutants

Cloning, expression, and purification

Because the chimeric variants did not provide enough clues into the stereoselectivity of PON1, we decided to make single mutants of 1-I-F11 to understand the effects of each individual amino acid. All single point mutants were cloned using site-directed mutagenesis and sent for sequencing. All sequences were confirmed and then expressed and purified. As with the chimeric mutants, no significant changes were seen in protein production and yield.

Kinetics

Table 4.2: CMP activity summary of 1-I-F11 single point variants

Mutant	S_P-CMP ($mM^{-1}min^{-1}$)	Racemic CMP ($mM^{-1}min^{-1}$)	R_P-CMP ($mM^{-1}min^{-1}$)	R_P/S_P
1-I-F11	4200	15000	11000	2.7
1-I-F11-L291I	530	3200	2700	5.1
1-I-F11-R134H	760	5600	4800	6.3
1-I-F11-M222F	760	5200	4590	6.1
1-I-F11 BLANK	27.2	300	272.8	10.0
1-I-F11-S332T	208	2300	2100	10.1
1-I-F11-A115H	45.8	650	650	14.2
1-I-F11-M55L	355	7000	6900	19.4
1-I-F11-V69L	140	3400	3260	23
G3C9	3.8	1020	1020	270

Table 4.2 illustrates the activities of G3C9, 1-I-F11, and single point mutants of 1-I-F11. Overall, all 7 mutations appear to have a significant effect on the racemic CMP activity of PON1. However, not much of a difference in the ratio of R_P-CMP to S_P-CMP activity was seen in some of the mutants. In particular, mutations M55L and V69L had the most drastic decrease in this ratio, and thus are most likely the mutations with the

largest impact on the stereoselectivity of 1-I-F11.

Molecular docking of CMP

Figure 4.7: Molecular docking of S_P-CMP in G3C9 active site

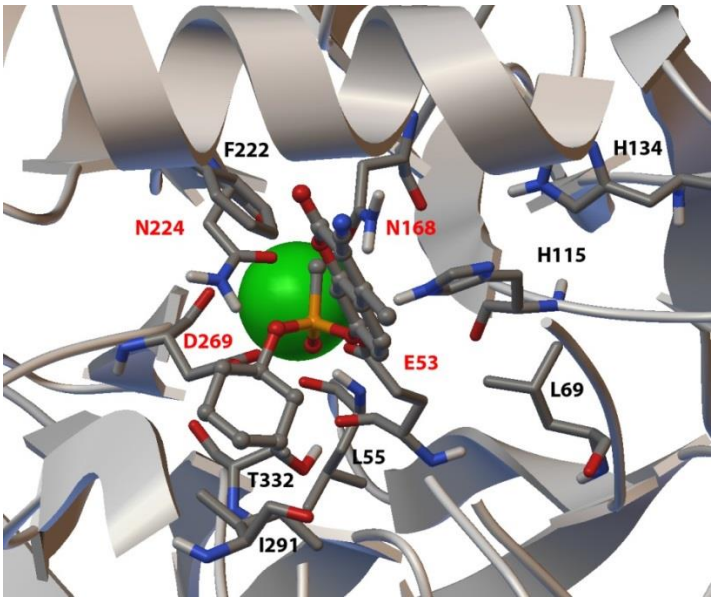
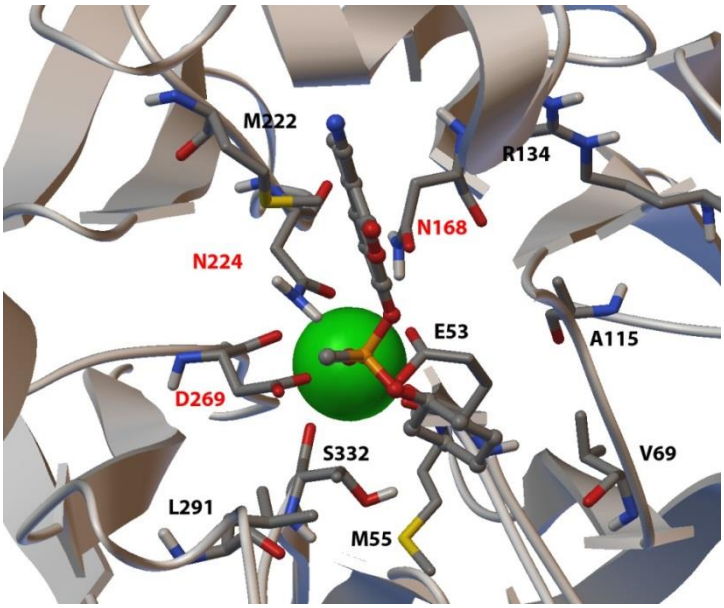


Figure 4.8: Molecular docking of S_P-CMP in 1-I-F11 active site



Based upon these images, molecular docking studies suggest that R134, L291, and S332 are the mutations most accountable for the reversion of stereoselectivity of 1-I-

F11. They allow for a more stable binding of CMP to the active site calcium in an anti-conformation with the theorized active site nucleophile, D269. These results, however, go against the results found in the kinetics summary above.

Summary and Future Directions

In this project, we attempted to understand the stereoselectivity of 1-I-F11with the end goal of improving PON1's efficacy as a bioscavenger. The data obtained from the chimeric variants indicated that both the N-terminal and C-terminal mutations were important for the stereoselectivity of the enzyme with the 4 n-terminal mutations having more of an impact on the overall activity towards CMP and S_P-CMP. With somewhat inconclusive results, we decided to make a series of single mutants reverting the mutations in 1-I-F11 back to the amino acid found in G3C9. These results showed that of the seven mutations, L55M and L69V were most important in the stereoselectivity of 1-I-F11. At this point, we are attempting to construct a series of single mutants of G3C9, performing the opposite process of the 1-I-F11 single mutants. Here, we will be converting the G3C9 residues into those found in 1-I-F11 and looking for which mutations produce the high stereoselectivity for S_P-CMP. In addition, we would like to construct a library randomizing position 71 to see how mutations at this position can affect the activity of PON1. Y71 is known to be influential in substrate binding due to its positing over the opening into the active site. Also, at the moment thermal stabilities are being conducted to understand the stability effects of the mutations in 1-I-F11.

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